Tetrahedron 68 (2012) 2851–2857

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

# Anti-inflammatory cyclopeptides from the marine sponge *Theonella swinhoei*

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ARTICLE INFO

*Article history:*
Received 7 November 2011
Received in revised form 11 January 2012
Accepted 31 January 2012
Available online 6 February 2012

*Keywords:*
Marine compounds
Cyclopeptides
*Theonella swinhoei*
Anti-inflammatory activity

ABSTRACT

DCCC chromatography followed by HPLC purification on the polar extract of marine sponge *Theonella swinhoei* resulted in the isolation of five new cyclopeptides, perthamides G–K. The new structures, featuring unprecedented amino acid units, were determined by interpretation of extensive spectroscopic and spectrometric data (MS, $^{1}$H and $^{13}$C NMR, COSY, HSQC, HMBC, and ROESY). Pharmacological analysis demonstrated that these natural cyclopeptides are endowed with anti-inflammatory potential as assessed by their ability to reduce carrageenan-induced mouse paw oedema.



## 1. Introduction

Cyclic peptides and cyclodepsipeptides from sponges have been extensively studied for their significant biological activities and structurally unique features incorporating several modified amino acid residues.[1,2] Whereas many of these compounds were shown to be antifungal, antiviral and antiproliferative, few examples of anti-inflammatory peptides from sponges or, more generally, from marine habitats have so far been reported.[3–6]

As a part of our systematic study on secondary metabolites from marine organisms collected at Solomon Islands,[7] we found a single specimen of the sponge *Theonella swinhoei* as an extraordinary source of new metabolites,[8–12] among which were identified two classes of anti-inflammatory peptides, perthamides C–F (Fig. 1),[13–16] and solomonamides.[17] Perthamide C (**1**, Fig. 1), the most abundant component of the sponge's polar extracts, is an octapeptide with an unprecedented primary structure. Six of its eight residues are non conventional amino acids: γ-methylproline, $N^{\delta}$-carbamoyl-β-$OSO_3$asparagine ($N^{\delta}$-c-β-OHAsn), *o*-tyrosine, dAbu, *O*-methylthreonine, and the β-amino acid AHMHA (3-amino-2-hydroxy-6-methylheptanoic acid).

From a biological point of view, perthamide C showed promising anti-inflammatory activity measured as a dose-dependent reduction of mouse carrageenan-induced paw oedema and as a down-regulation of TNF-α and IL-8 release, two key biomarkers in the inflammatory response of primary human keratinocytes cells.[16]

In order to re-isolate further amounts of perthamide C for additional pharmacological studies, we re-examined the polar extracts of the sponge *T. swinhoei*, still available in our laboratories. In this paper we report the isolation of perthamide C and five new minor analogues, perthamides G–K (**2**–**6**), whose structures have been determined by NMR spectroscopy and mass spectrometry analysis (Fig. 2).

## 2. Results and discussion

The initial processing of the *T. swinhoei* (coll. No. R3170) was conducted according to procedures described previously.[13] The *n*-BuOH extract was purified by DCCC (*n*-BuOH/$Me_2CO$/$H_2O$, descending mode) followed by reverse-phase HPLC using a C-12 column (MeOH/$H_2O$ 59% with 0.1% TFA as eluent) to afford 69 mg of perthamide C (**1**), 10.8 mg of perthamide G (**2**), 17.8 mg of perthamide H (**3**), 16.6 mg of perthamide I (**4**), 12.2 mg of perthamide J (**5**), and 16.2 mg of perthamide K (**6**).

The HR-ESIMS spectrum of perthamide G (**2**) showed a major peak at *m*/*z* 1052.4046 $[M-Na]^-$ corresponding to a molecular formula $C_{43}H_{62}N_{11}NaO_{18}S$, 14 u.m.a. less than perthamide C.

Accordingly, comparison of the $^{1}$H NMR spectrum of perthamide G (**2**) with that of parent compound perthamide C (**1**)

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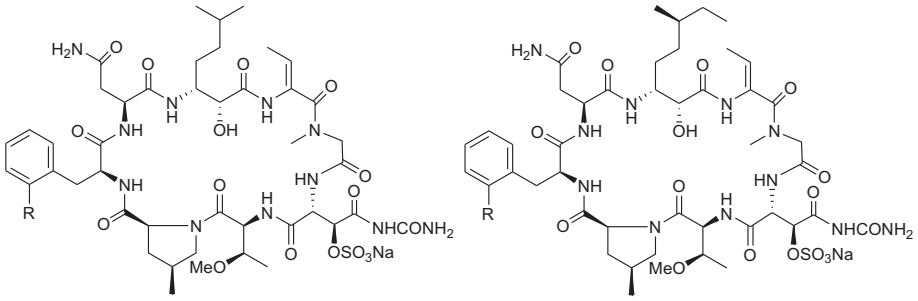
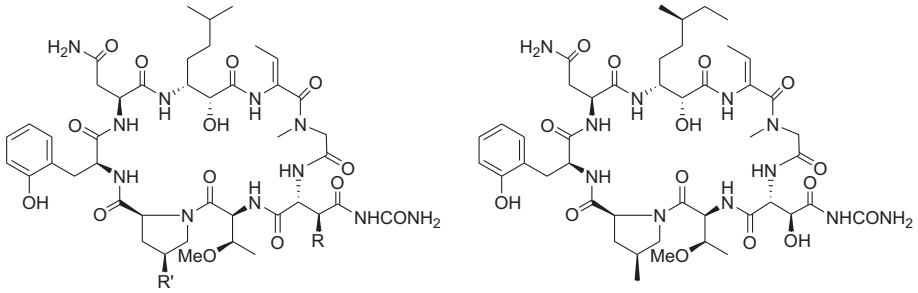
doi:10.1016/j.tet.2012.01.097



Perthamide C (**1**) R = OH
Perthamide D R = H

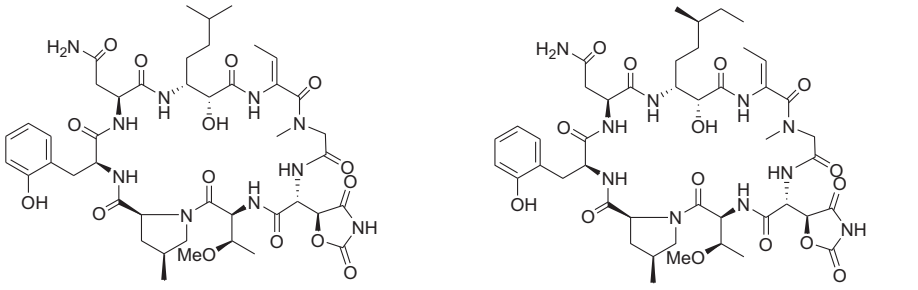
Perthamide E R = OH
Perthamide F R = H

**Fig. 1.** Perthamides C–F previously isolated from *Theonella swinhoei*.



Perthamide G (**2**) R = $OSO_3Na$ R' = H
Perthamide H (**3**) R = OH R' = $CH_3$

Perthamide I (**4**)



Perthamide J (**5**)

Perthamide K (**6**)

**Fig. 2.** New perthamide derivatives from *T. swinhoei*.

showed the absence of the methyl group signal at $\delta_H$ 1.04 assigned to 3-Me group in the γ-MePro residue. One spin system of the type X–CH–$CH_2$–$CH_2$–$CH_2$–X′ was easily defined using TOCSY, COSY, HSQC and HMBC data and indicated the presence of one proline unit. The remaining seven amino acid units were found to be the same of perthamide C, as suggested by 2D NMR spectroscopic data.

Marfey's method revealed L configuration for the Pro residue, whereas the relative and absolute configuration of L-Asn, L-*o*-Tyr, L-ThrOMe, *erythro*-D-$N^{\delta}$-c-β-OHAsn, (2*R*,3*R*)-AHMHA and *Z*-dAbu, were determined by comparison of NMR spectroscopic data and LC–MS analysis of the L/D-FDAA-derivatized hydrolysate of **2** with **1**.

The molecular formula of perthamide H (**3**), $C_{44}H_{65}N_{11}O_{15}$, was consistent with the lack of the sulfate group in the $N^{\delta}$-carbamoyl-β-$OSO_3$asparagine residue.

This hypothesis was confirmed by analysis of the NMR spectroscopic data. An additional exchangeable proton signal at $\delta_H$ 6.16 (br d, $J$=6.4) was observed in the $^1$H NMR spectrum of perthamide H (**3**), and was assigned to the β-OH group in the $N^{\delta}$-c-β-OHAsn residue on the basis of the COSY spin system NH/H-2/H-3/OH and the diagnostic HMBC correlations from the hydroxyl proton to C-2, C-3 and C-4 (Fig. 3). The downfield shift exhibited by the $NH^{\delta}$ proton (9.52 in **3** vs 8.90 in **1**) and C-2 (54.8 in **3** vs 53.0 in **1**) and C-4 (173.2 in **3** vs 170.5 in **1**) carbons and the upfield shift of H-2, H-3 and C-3 nuclei (Table 1) were all compatible with the presence of the $N^{\delta}$-c-β-OHAsn residue in **3**.

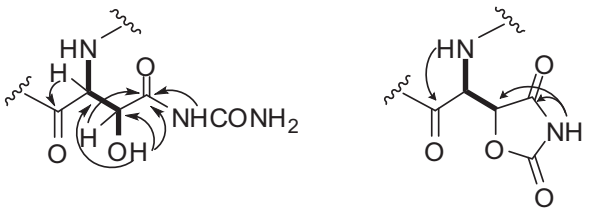


**Fig. 3.** $N^{\delta}$-c-β-OHAsn and ADAA residues in perthamides H and J, respectively, with COSY connectivities (bold bonds) and HMBC correlations (arrows).

The HR-ESIMS of perthamide I (**4**) showed an ion peak at $m/z$ 1000.4784 $[M-H]^-$ ($m/z$ 1000.4740, calcd for $C_{45}H_{66}N_{11}O_{15}$), 14 mass units higher than that of **3**. The NMR data for **4** (Table 2)

**Table 1**
NMR data (700 MHz, DMSO-$d_6$) of perthamide C (**1**) and perthamides G–H (**2** and **3**)

| Perthamide C (**1**) | | | Perthamide G (**2**) | | | Perthamide H (**3**) | | |
|---|---|---|---|---|---|---|---|---|
| AA | $\delta_H$[a] | $\delta_C$ | AA | $\delta_H$[a] | $\delta_C$ | AA | $\delta_H$[a] | $\delta_C$ |
| *ThrOMe* | | | *ThrOMe* | | | *ThrOMe* | | |
| 1 | — | 171.0 | 1 | — | 171.1 | 1 | — | 170.9 |
| 2 | 4.92 d (9.5) | 55.3 | 2 | 4.93 d (9.3) | 55.4 | 2 | 4.95 d (9.7) | 55.3 |
| 3 | 4.16 ovl | 72.8 | 3 | 4.15 ovl | 73.0 | 3 | 4.15 ovl | 72.9 |
| 4 | 1.24 d (5.8) | 14.3 | 4 | 1.23 d (6.0) | 14.3 | 4 | 1.17 d (6.0) | 14.0 |
| OMe | 3.27 s | 54.7 | OMe | 3.25 s | 54.7 | OMe | 3.26 s | 54.7 |
| NH | 9.05 d (8.8) | | NH | 8.94 d (8.2) | | NH | 9.03 d (9.5) | |
| *γMePro* | | | *MePro* | | | *γMePro* | | |
| 1 | — | 170.7 | 1 | — | 170.9 | 1 | — | 170.8 |
| 2 | 3.90 dd (11.0, 6.7) | 63.4 | 2 | 3.88 dd (9.4, 7.7) | 62.9 | 2 | 3.89 dd (11.2, 6.6) | 63.4 |
| 3 | 0.68–0.74 m, 2.07–2.11 m | 36.1 | 3 | 1.13 ovl, 2.06–2.11 m | 28.2 | 3 | 0.68–0.74 m, 2.08–2.11 m | 36.1 |
| 4 | 2.29–2.22 m | 33.2 | 4 | 1.81–1.88 m, 1.93 m | 25.1 | 4 | 2.22–2.29 m | 33.2 |
| 5 | 3.40 t (10.8), 4.11 ovl | 53.0 | 5 | 3.79–3.85 m, 3.94 t (9.2) | 46.8 | 5 | 3.40 t (10.8), 4.12 ovl | 53.0 |
| 6 | 1.04 d (6.1) | 15.9 | | | | 6 | 1.04 d (6.4) | 15.8 |
| *o-Tyr* | | | *o-Tyr* | | | *o-Tyr* | | |
| 1 | — | 170.6 | 1 | — | 170.6 | 1 | — | 170.7 |
| 2 | 4.12 ovl | 56.3 | 2 | 4.12 ovl | 56.4 | 2 | 4.11 ovl | 56.4 |
| 3 | 2.86 dd (13.2, 3.2), 2.95 t (13.2) | 30.6 | 3 | 2.84 dd (13.5, 3.7), 2.94 t (13.5) | 30.7 | 3 | 2.86 dd (13.2, 3.5), 2.94 t (13.2) | 30.6 |
| 1′ | — | 124.0 | 1′ | — | 124.4 | 1′ | — | 124.3 |
| 2′ | — | 154.4 | 2′ | — | 154.6 | 2′ | — | 154.5 |
| 3′ | 6.88 d (7.8) | 114.5 | 3′ | 6.84 d (8.0) | 114.4 | 3′ | 6.88 d (7.9) | 114.5 |
| 4′ | 7.11 t (7.8) | 127.9 | 4′ | 7.08 t (8.0) | 128.0 | 4′ | 7.10 t (7.8) | 127.9 |
| 5′ | 6.80 t (7.4) | 119.7 | 5′ | 6.78 t (7.3) | 119.8 | 5′ | 6.80 t (7.4) | 119.8 |
| 6′ | 7.16 d (7.4) | 130.3 | 6′ | 7.12 d (7.3) | 130.3 | 6′ | 7.16 d (7.4) | 130.4 |
| NH | 7.19 ovl | | NH | 7.26 ovl | | NH | 7.22 d (6.7) | |
| OH | 10.2 s | | OH | 10.1 s | | OH | 10.2 s | |
| *Asn* | | | *Asn* | | | *Asn* | | |
| 1 | — | 170.3 | 1 | — | 170.4 | 1 | — | 170.5 |
| 2 | 4.68–4.72 m | 48.0 | 2 | 4.68–4.71 m | 48.1 | 2 | 4.68–4.72 m | 48.0 |
| 3 | 2.41 dd (16.9, 2.2), 3.15 d (16.9) | 36.9 | 3 | 2.41 dd (17.2, 3.1), 3.10 dd (17.2, 4.3) | 36.9 | 3 | 2.40 dd (17.5, 2.9), 3.15 dd (17.5, 3.9) | 36.9 |
| 4 | — | 172.5 | 4 | — | 172.6 | 4 | — | 172.4 |
| NH | 7.18 ovl | | NH | 7.16 ovl | | NH | 7.18 d (7.1) | |
| $NH_2$ | 6.51 br s, 7.86 br s | | $NH_2$ | 6.51 s, 7.80 s | | $NH_2$ | 6.70 br s, 7.82 br s | |
| *AHMHA* | | | *AHMHA* | | | *AHMHA* | | |
| 1 | — | 169.7 | 1 | — | 169.9 | 1 | — | 169.9 |
| 2 | 4.14 ovl | 72.6 | 2 | 4.14 ovl | 72.7 | 2 | 4.14 ovl | 72.6 |
| 3 | 4.00 t (9.9) | 52.0 | 3 | 3.98 t (10.2) | 51.9 | 3 | 3.99 t (9.9) | 52.0 |
| 4 | 1.16 ovl, 1.41 ovl | 24.1 | 4 | 1.14 ovl, 1.39 ovl | 24.2 | 4 | 1.15 ovl, 1.41 ovl | 24.2 |
| 5 | 0.93–0.98 m, 1.15 ovl | 34.4 | 5 | 0.93–0.98 m, 1.15 ovl | 34.3 | 5 | 0.93–0.98 m, 1.16 ovl | 34.4 |
| 6 | 1.41 ovl | 27.2 | 6 | 1.40 ovl | 27.1 | 6 | 1.41 ovl | 27.2 |
| 7 | 0.77 d (6.4) | 21.9 | 7 | 0.76 d (6.6) | 21.8 | 7 | 0.77 d (6.5) | 21.9 |
| 8 | 0.78 d (6.4) | 22.2 | 8 | 0.77 d (6.6) | 22.4 | 8 | 0.78 d (6.5) | 22.0 |
| NH | 6.38 d (9.1) | | NH | 6.40 d (9.5) | | NH | 6.36 d (9.3) | |
| OH | 4.81 br s | | OH | 4.81 br s | | OH | 4.83 br s | |
| *dAbu* | | | *dAbu* | | | *dAbu* | | |
| 1 | — | 167.2 | 1 | — | 167.4 | 1 | — | 167.3 |
| 2 | — | 132.1 | 2 | — | 132.3 | 2 | — | 132.3 |
| 3 | 5.77 q (6.9) | 126.4 | 3 | 5.75 q (6.8) | 126.3 | 3 | 5.77 q (6.9) | 126.4 |
| 4 | 1.54 d (6.9) | 12.1 | 4 | 1.55 d (6.8) | 12.1 | 4 | 1.55 d (6.9) | 12.1 |
| NH | 8.65 s | | NH | 8.60 s | | NH | 8.68 s | |
| *NMeGly* | | | *NMeGly* | | | *NMeGly* | | |
| 1 | — | 167.1 | 1 | — | 167.2 | 1 | — | 167.3 |
| 2 | 3.31 d (17.9), 4.21 d (17.9) | 51.4 | 2 | 3.30 ovl, 4.20 d (17.8) | 51.4 | 2 | 3.36 ovl, 4.25 d (17.4) | 51.4 |
| NMe | 2.70 s | 34.4 | NMe | 2.70 s | 34.5 | NMe | 2.70 s | 34.4 |
| *$N^{\delta}$-c-β-$OSO_3$Asn* | | | *$N^{\delta}$-c-β-$OSO_3$Asn* | | | *$N^{\delta}$-c-β-OHAsn* | | |
| 1 | — | 167.4 | 1 | — | 167.5 | 1 | — | 168.4 |
| 2 | 5.05 t (7.4) | 53.0 | 2 | 5.03 t (7.4) | 53.0 | 2 | 4.88 t (7.6) | 54.8 |
| 3 | 4.59 d (7.1) | 74.9 | 3 | 4.58 d (7.1) | 74.9 | 3 | 4.12 ovl | 71.3 |
| 4 | — | 170.5 | 4 | — | 170.7 | 4 | — | 173.2 |
| βNH | 7.18 ovl | | βNH | 7.15 ovl | | βNH | 7.08 d (7.6) | |
| NH | 8.90 s | | NH | 8.87 br s | | NH | 9.52 s | |
| CO | — | n.o | CO | — | n.o | CO | — | n.o |
| $NH_2$ | 7.31 br s, 7.51 br s | | $NH_2$ | 7.30 br s, 7.50 br s | | $NH_2$ | 7.31 br s, 7.51 br s | |
| | | | | | | OH | 6.16 br d (6.4) | |

[a] Coupling constants are in parentheses and given in hertz. Ovl: overlapped; n.o: not observed. $^1H$ and $^{13}C$ assignments aided by COSY, TOCSY, HSQC and HMBC experiments.

closely resembled those of **3** with the exception that resonances belonging to the β-amino acid AHMHA residue were replaced by resonances belonging to a 3-amino-2-hydroxy-6-methyloctanoic acid (AHMOA) unit as in perthamide E.[16]

LC–MS analysis of the L-FDAA-derivatized hydrolysates of **3** and **4**, and the NMR data revealed all α-amino acid residues to possess configurations identical to those in **1**. On the basis of closely matching NMR data, the absolute configuration of AHMOA unit in **4**

**Table 2**
NMR data (700 MHz, DMSO-$d_6$) of perthamides I–K (**4**–**6**)

| Perthamide I (**4**) | | | Perthamide J (**5**) | | | Perthamide K (**6**) | | |
|---|---|---|---|---|---|---|---|---|
| AA | $\delta_H$[a] | $\delta_C$ | AA | $\delta_H$[a] | $\delta_C$ | AA | $\delta_H$[a] | $\delta_C$ |
| *ThrOMe* | | | *ThrOMe* | | | *ThrOMe* | | |
| 1 | — | 171.0 | 1 | — | 171.1 | 1 | — | 170.8 |
| 2 | 4.96 d (9.6) | 55.2 | 2 | 5.00 dd (1.5, 9.7) | 55.5 | 2 | 5.01 dd (1.5, 9.7) | 55.4 |
| 3 | 4.15 ovl | 72.8 | 3 | 4.14–4.17 m | 72.6 | 3 | 4.16 ovl | 72.7 |
| 4 | 1.18 d (6.0) | 14.3 | 4 | 1.14 d (6.1) | 14.1 | 4 | 1.15 d (6.2) | 14.0 |
| OMe | 3.27 s | 54.7 | OMe | 3.25 s | 54.9 | OMe | 3.27 s | 54.8 |
| NH | 9.02 d (9.5) | | NH | 8.99 d (9.7) | | NH | 8.99 d (9.7) | |
| *γMePro* | | | *γMePro* | | | *γMePro* | | |
| 1 | — | 170.9 | 1 | — | 170.8 | 1 | — | 170.7 |
| 2 | 3.91 dd (11.1, 6.5) | 63.1 | 2 | 3.86 dd (11.2, 6.6) | 63.5 | 2 | 3.91 dd (11.0, 6.6) | 63.2 |
| 3 | 0.71–0.73 m, 2.08–2.14 m | 36.2 | 3 | 0.68–0.75 m, 2.09–2.13 m | 36.1 | 3 | 0.63–0.71 m, 2.09–2.13 m | 36.0 |
| 4 | 2.22–2.29 m | 33.3 | 4 | 2.20–2.27 m | 33.3 | 4 | 2.22–2.29 m | 33.3 |
| 5 | 3.40 t (10.8), 4.12 ovl | 53.2 | 5 | 3.40 ovl, 4.19 dd (10.3, 7.5) | 53.0 | 5 | 3.40 ovl, 4.18 dd (10.4, 7.6) | 52.9 |
| 6 | 1.05 d (6.3) | 15.7 | 6 | 1.04 d (6.4) | 15.8 | 6 | 1.04 d (6.2) | 15.6 |
| *o-Tyr* | | | *o-Tyr* | | | *o-Tyr* | | |
| 1 | — | 170.7 | 1 | — | 170.7 | 1 | — | 170.5 |
| 2 | 4.11 ovl | 56.5 | 2 | 4.11 ovl | 56.4 | 2 | 4.10 ovl | 56.2 |
| 3 | 2.89 dd (13.2, 3.6), 2.95 t (13.2) | 30.7 | 3 | 2.84 dd (13.0, 3.4), 2.93 t (13.0) | 30.5 | 3 | 2.87 dd (12.8, 3.4), 2.95 t (12.8) | 30.2 |
| 1′ | — | 124.3 | 1′ | — | 124.0 | 1′ | — | 124.1 |
| 2′ | — | 154.6 | 2′ | — | 154.4 | 2′ | — | 154.5 |
| 3′ | 6.88 d (7.9) | 114.5 | 3′ | 6.88 d (8.0) | 114.5 | 3′ | 6.85 d (8.0) | 114.4 |
| 4′ | 7.11 t (7.8) | 128.1 | 4′ | 7.12 t (8.0) | 128.1 | 4′ | 7.12 t (8.0) | 128.2 |
| 5′ | 6.80 t (7.4) | 119.9 | 5′ | 6.79 t (7.3) | 119.9 | 5′ | 6.80 t (7.3) | 120.0 |
| 6′ | 7.18 d (7.4) | 130.6 | 6′ | 7.16 d (7.3) | 130.4 | 6′ | 7.16 d (7.3) | 130.3 |
| NH | 7.22 d (6.7) | | NH | 7.21 d (6.6) | | NH | 7.21 d (6.6) | |
| OH | 10.1 s | | OH | 10.2 s | | OH | 10.1 s | |
| *Asn* | | | *Asn* | | | *Asn* | | |
| 1 | — | 170.5 | 1 | — | 170.6 | 1 | — | 170.6 |
| 2 | 4.68–4.73 m | 48.0 | 2 | 4.68–4.72 m | 47.7 | 2 | 4.68–4.74 m | 47.7 |
| 3 | 2.41 dd (17.4, 3.0), 3.15 dd (17.4, 3.9) | 36.8 | 3 | 2.39 dd (17.5, 3.2), 3.18 dd (17.5, 4.2) | 36.8 | 3 | 2.41 dd (17.6, 3.2), 3.20 dd (17.6, 4.2) | 36.6 |
| 4 | — | 172.6 | 4 | — | 173.0 | 4 | — | 173.1 |
| NH | 7.18 d (7.1) | | NH | 7.14 d (7.8) | | NH | 7.14 d (7.8) | |
| $NH_2$ | 6.70 br s, 7.79 br s | | $NH_2$ | 6.63 s, 8.02 s | | $NH_2$ | 6.63 s, 8.00 s | |
| *AHMOA* | | | *AHMHA* | | | *AHMOA* | | |
| 1 | — | 169.7 | 1 | — | 169.4 | 1 | — | 169.7 |
| 2 | 4.13 ovl | 72.8 | 2 | 4.12 ovl | 72.4 | 2 | 4.14 ovl | 72.5 |
| 3 | 4.02 t (9.8) | 52.0 | 3 | 3.94 t (10.6) | 52.2 | 3 | 4.01 t (9.7) | 51.8 |
| 4 | 1.07–1.11 m, 1.42–1.48 m | 23.6 | 4 | 1.15 ovl, 1.40 ovl | 23.8 | 4 | 1.07–1.11 m, 1.43–1.49 m | 23.7 |
| 5 | 1.05 ovl | 31.7 | 5 | 0.92–0.96 m, 1.17 ovl | 34.2 | 5 | 1.06 ovl | 31.7 |
| 6 | 1.17 ovl | 33.3 | 6 | 1.40 ovl | 27.1 | 6 | 1.18 ovl | 33.4 |
| 7 | 1.03 ovl, 1.22 ovl | 28.5 | 7 | 0.76 d (6.5) | 21.8 | 7 | 1.03 ovl, 1.21 ovl | 28.5 |
| 8 | 0.77 t (7.1) | 11.1 | 8 | 0.77 d (6.5) | 22.2 | 8 | 0.78 t (7.1) | 11.0 |
| 9 | 0.75 d (6.1) | 18.6 | | | | 9 | 0.75 d (6.2) | 18.6 |
| NH | 6.36 d (9.0) | | NH | 6.35 d (9.3) | | NH | 6.36 d (9.2) | |
| OH | 4.83 br s | | OH | 4.92 s | | OH | 4.82 br s | |
| *dAbu* | | | *dAbu* | | | *dAbu* | | |
| 1 | — | 167.5 | 1 | — | 167.3 | 1 | — | 167.3 |
| 2 | — | 132.3 | 2 | — | 132.2 | 2 | — | 132.1 |
| 3 | 5.78 q (6.9) | 126.6 | 3 | 5.81 q (6.7) | 126.4 | 3 | 5.81 q (6.6) | 126.4 |
| 4 | 1.56 d (6.9) | 11.9 | 4 | 1.59 d (7.0) | 12.1 | 4 | 1.56 d (7.0) | 12.1 |
| NH | 8.66 s | | NH | 8.69 s | | NH | 8.68 s | |
| *NMeGly* | | | *NMeGly* | | | *NMeGly* | | |
| 1 | — | 167.2 | 1 | — | 168.3 | 1 | — | 168.3 |
| 2 | 3.36 ovl, 4.28 d (17.4) | 51.6 | 2 | 3.53 d (18.0), 4.26 d (18.0) | 51.4 | 2 | 3.53 d (18.0), 4.26 d (18.0) | 51.5 |
| NMe | 2.70 s | 34.4 | NMe | 2.83 s | 34.9 | NMe | 2.71 s | 34.5 |
| *$N^{\delta}$-c-β-OHAsn* | | | *ADAA* | | | *ADAA* | | |
| 1 | — | 168.4 | 1 | — | 166.1 | 1 | — | 166.1 |
| 2 | 4.89 t (7.7) | 54.7 | 2 | 5.02 dd (5.5, 3.8) | 52.5 | 2 | 5.05 dd (5.6, 3.7) | 52.4 |
| 3 | 4.12 ovl | 71.1 | 3 | 5.24 d (3.8) | 79.5 | 3 | 5.22 d (3.7) | 79.3 |
| 4 | — | 173.0 | 4 | — | 154.9 | 4 | — | 154.9 |
| βNH | 7.09 d (7.7) | | βNH | 7.78 d (5.5) | | βNH | 7.80 d (5.6) | |
| NH | 9.48 s | | NH | 12.3 s | | NH | 12.3 s | |
| CO | — | n.o | CO | — | n.o | CO | — | n.o |
| $NH_2$ | 7.29 br s, 7.52 br s | | | | | | | |
| OH | 6.14 br s | | | | | | | |

[a] Coupling constants are in parentheses and given in hertz. Ovl: overlapped; n.o: not observed. $^1H$ and $^{13}C$ assignments aided by COSY, TOCSY, HSQC and HMBC experiments.

(Fig. 2) was assumed to be the same as the corresponding residue in perthamide E (Fig. 1), recently proposed through enantioselective synthesis[14] and quantum chemical calculation of NMR chemical shifts.[16]

The HR-ESIMS spectrum of perthamide J (**5**) showed an ion peak at *m/z* 969.4358 [M−H], corresponding to a molecular formula of $C_{44}H_{62}N_{10}O_{15}$, which is 97 u.m.a. less than perthamide C. NMR data of perthamide J (**5**) were almost superimposable to those of parent peptide, whereas only a perturbation in the $N^{\delta}$-c-β-$OSO_3$Asn residue was observed (Table 2).

In particular in the $^1$H NMR spectrum the signals relative to the terminal $-NH_2$ group (7.31/7.51) in the δ-carbamoyl moiety were absent. The downfield shift shown by both the proton and carbon in the position β ($\delta_H$ 5.24 vs 4.59 in **1**; $\delta_C$ 79.5 vs 74.9 in **1**) was indicative of an acylation. As shown in Fig. 3, the signal of the $NH^{\delta}$, downfield shifted at $\delta_H$ 12.3 versus 8.90 in perthamide C (**1**), showed HMBC correlations with C-3 ($\delta_C$ 79.5) and C-4 ($\delta_C$ 154.9). No further acyl group could be revealed by analysis of the NMR spectra;[18] however MS data and chemical shift consideration clearly suggested a cyclization between the β-OH group and the carbamoyl moiety to form a cyclic oxazolidin-2,4-dione. Thus, perthamide J (**5**) contained the new 2-amino-2-(2,4-dioxooxazolidin-5-yl)acetic acid residue (ADAA), replacing the $N^{\delta}$-c-β-$OSO_3$Asn residue in perthamide C.

Comparison by LC−MS of the L/D-FDAA derivatives of the β-OHAsp residue obtained after acid hydrolysis of **5** with the corresponding derivatives from the hydrolysate of an authentic sample of perthamide C (**1**) showed that the 2-amino-2-(2,4-dioxooxazolidin-5-yl)acetic acid residue has the same (2*R*,3*S*) configuration as established for the $N^{\delta}$-c-β-$OSO_3$Asn residue in perthamide C (**1**) through stereoselective synthesis and Marfey analysis of all stereoisomers of β-hydroxyaspartate.[13]

The molecular formula of perthamide K (**6**), $C_{45}H_{64}N_{10}O_{15}$, with an additional methylene group as compared to perthamide J (**5**), was deduced by HR-ESIMS, *m/z* 983.4520 $[M-H]^-$. A detailed analysis of the NMR data of **6** clearly established that its amino acid sequence was identical to **5**, except for the occurrence of a 3-amino-2-hydroxy-6-methyloctanoic acid (AHMOA) residue instead of a 3-amino-2-hydroxy-6-methylheptanoic acid (AHMHA) residue.

Through careful analysis of HMBC and ROESY data, the amino acid sequence, shown in Fig. 4 for perthamide J (**5**), was proved in perthamides G (**2**), H (**3**), I (**4**) and K (**6**) to be the same as found in **1**.

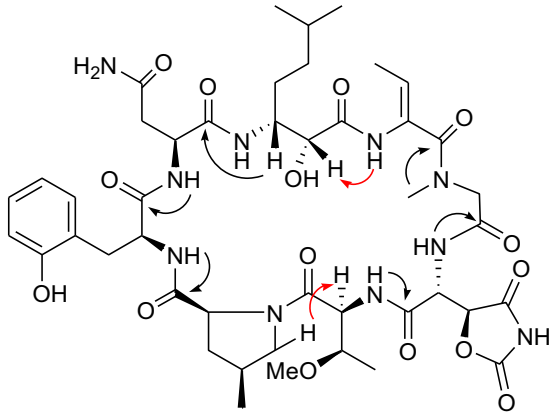



**Fig. 4.** Selected ROE (red arrows) and HMBC (black arrows) correlations for perthamide J (**5**).

To rule out the suspect of a-biotic origin of perthamides H−K (**3**–**6**) from the parent compounds perthamides C and E during HPLC isolation procedures in which MeOH−$H_2O$/TFA (0.1%) was used as the elution solvent, the crude *n*-BuOH extract of the sponge was analyzed by LC−ESIMS in non-acidic conditions. As shown in Fig. 5, the detection of the ions corresponding to the $MH^+$ of perthamides H, I, J and K at *m/z* 988.4, 1002.4, 971.4, 985.4, respectively (positive ionization mode), in the LC−ESIMS run strongly supported the natural origin of perthamides H−K (**3**–**5**).

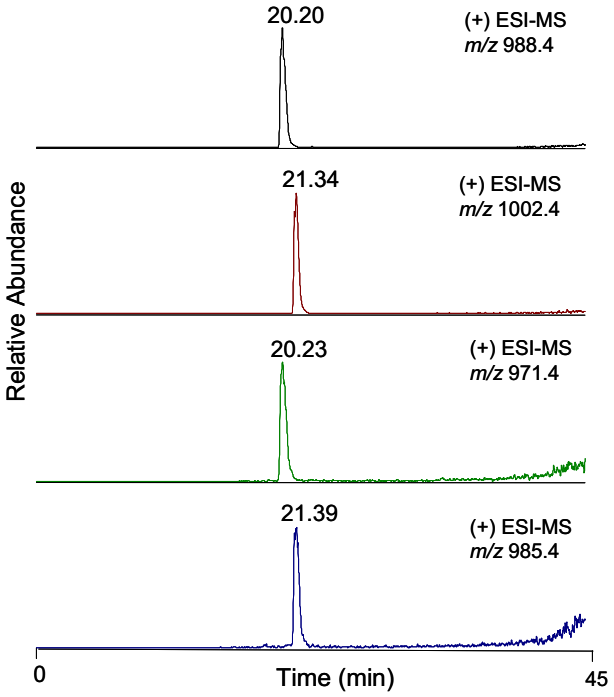



**Fig. 5.** Extract ions of perthamides H (black line), I (red line), J (green line) and K (blue line) in the LC−ESIMS run of crude *n*-BuOH extract.

We have previously reported that perthamide C (**1**) significantly reduced carrageenan-induced mouse paw oedema in both the early phase (0–6 h) and the late phase (24–96 h).[13] In particular, perthamide C (**1**) displayed a dose-dependent (0.1, 0.3 and 1 mg/kg) anti-inflammatory activity causing about 60% reduction of oedema development at the dose of 300 μg/kg.[13]

With this background in mind, we tested all new derivatives in carrageenan-induced mouse paw oedema. As reported in Fig. 6A, perthamides H (**3**), I (**4**) and K (**6**) when administered intraperitoneally at the dose of 0.3 mg/kg, showed the same behaviour as perthamide C (**1**) reducing mouse oedema in both early phase (0–6 h) and in the late phase (24–96 h). Notably, Fig. 6B, perthamide G (**2**), with a proline replacing the γ-methylproline residue in perthamide C (**1**), is not endowed with anti-inflammatory activity whereas perthamide J (**5**), with the ADAA residue, is the most efficacious molecule of this small library. All these data pointed towards the pharmacophoric rule played by the simultaneous presence in the molecule of γ-MePro, AHMHA and ADAA units for anti-inflammatory activity.

In conclusion, in this paper we report the isolation and the structural characterization of five new perthamide C derivatives, perthamides G–K (**2**–**6**), from the marine sponge *T. swinhoei*. Pharmacological analysis demonstrated that these natural cyclopeptides are endowed with anti-inflammatory potential as assessed by their ability to reduce carrageenan-induced mouse paw oedema and allowed us to delineate a structure−activity relationship.

## 3. Experimental section

### 3.1. General experimental procedures

Specific rotations were measured on a Jasco P-2000 polarimeter. High-resolution ESIMS spectra were performed on Q-ToF Premiere

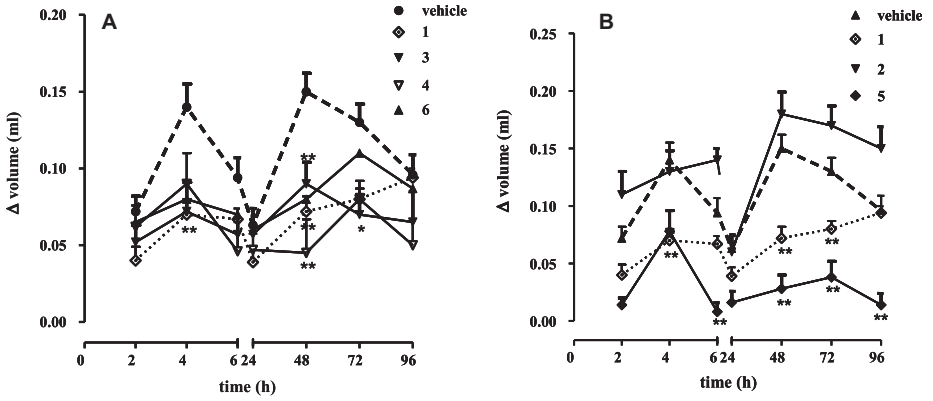



**Fig. 6.** Inhibition on carrageenan-induced paw oedema by perthamide C (**1**) and perthamides G–K (**2**–**6**) at 0.3 mg/kg dose.

(Waters Co.) mass spectrometer. LC–MS experiments were performed on an LCQ Deca (Thermo Scientific) mass spectrometer equipped with a FinniganMat quaternary pump. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers ($^1$H at 500 and 700 MHz, $^{13}$C at 125 and 175 MHz, respectively) equipped with Sun hardware, $\delta$ (ppm), $J$ in hertz, spectra referred to DMSO-$d_6$ as internal standard ($\delta_H$ 2.50, $\delta_C$ 39.5). HPLC was performed using a Waters 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

Through-space $^1$H connectivities were evidenced using a ROESY experiment with mixing time of 100 ms.

DCCC was performed using a DCC-A (Rakakikai Co. Di Tokio) equipped with 250 columns (internal diameter 3 mm).

The purities of compounds were determined to be greater than 95% by HPLC, MS and NMR.

### 3.2. Sponge material and separation of individual peptides

*T. swinhoei* (order Lithistida, family Theonellidae) was collected at a depth of 22 m, on an isolated reef off the western coast of Malaita Island, Solomon Islands, in July 2004. The samples were frozen immediately after collection and lyophilized to yield 400 g of dry mass. Taxonomic identification was performed by Dr. John Hooper at Queensland Museum, Brisbane, Australia, where a voucher specimen is deposited under the accessing number G322662.

The lyophilized material (400 g) was extracted with methanol (3×1.5 L) at room temperature and the crude methanolic extract was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/$H_2O$ containing 10% $H_2O$ and partitioned against *n*-hexane to obtain 15.2 g of the corresponding extract. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$ to obtain 5.83 g of the corresponding extract. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH to obtain 6.0 g of the corresponding extract.

The *n*-BuOH extract (4.0 g) was chromatographed in two runs by DCCC using *n*-BuOH/$Me_2CO$/$H_2O$ (3:1:5) in the descending mode (the upper phase was the stationary phase), flow rate 8 mL/min; 4 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Peptide containing fractions (421.2 mg) were purified in many runs by HPLC on a reverse-phase C-12 Jupiter Proteo column (Phenomenex, 4 μ, 250×4.6 mm, 1.0 mL/min), eluting in isocratic mode with 59% MeOH/$H_2O$ (0.1% TFA) to afford 10.8 mg of perthamide G (**2**) ($t_R$=6.3 min), 69.0 mg of perthamide C (**1**) ($t_R$=9.6 min), 17.8 mg of perthamide H (**3**) ($t_R$=11.7 min), 12.2 mg of perthamide J (**5**) ($t_R$=12.6 min), 67.8 mg of perthamide E ($t_R$=14 min), 16.6 mg of perthamide I (**4**) ($t_R$=19.5 min) and 16.2 mg of perthamide K (**6**) ($t_R$=20.4 min).

### 3.3. Characteristic data for each peptide

*3.3.1. Perthamide C (**1**).* White amorphous solid; $[\alpha]_D^{22}$ −87.7 (*c* 0.87, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 1; HR-ESIMS *m*/*z* 1066.4106 $[M-Na]^-$ (calcd for $C_{44}H_{64}N_{11}O_{18}S$ *m*/*z* 1066.4151).

*3.3.2. Perthamide G (**2**).* White amorphous solid; $[\alpha]_D^{22}$ −123.3 (*c* 0.15, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 1; HR-ESIMS *m*/*z* 1052.4046 $[M-Na]^-$ (calcd for $C_{43}H_{62}N_{11}O_{18}S$ *m*/*z* 1052.3995).

*3.3.3. Perthamide H (**3**).* White amorphous solid; $[\alpha]_D^{22}$ −12.9 (*c* 0.55, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 1; HR-ESIMS *m*/*z* 986.4629 $[M-H]^-$ (calcd for $C_{44}H_{64}N_{11}O_{15}$ *m*/*z* 986.4583).

*3.3.4. Perthamide I (**4**).* White amorphous solid; $[\alpha]_D^{22}$ −119.2 (*c* 0.13, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 2; HR-ESIMS *m*/*z* 1000.4787 $[M-H]^-$ (calcd for $C_{45}H_{66}N_{11}O_{15}$ *m*/*z* 1000.4740).

*3.3.5. Perthamide J (**5**).* White amorphous solid; $[\alpha]_D^{22}$ +227.7 (*c* 0.26, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 2; HR-ESIMS *m*/*z* 969.4358 $[M-H]^-$ (calcd for $C_{44}H_{61}N_{10}O_{15}$ *m*/*z* 969.4318).

*3.3.6. Perthamide K (**6**).* White amorphous solid; $[\alpha]_D^{22}$ −74.9 (*c* 0.37, $CH_3OH$); $^1$H and $^{13}$C NMR data in DMSO-$d_6$ are given in Table 2; HR-ESIMS *m*/*z* 983.4520 $[M-H]^-$ (calcd for $C_{45}H_{63}N_{10}O_{15}$ *m*/*z* 983.4474).

### 3.4. Determination of amino acid absolute configuration

Perthamides C, E and G–K (500 μg) were dissolved, in four evacuated glass tubes, in 6 N aq HCl (0.5 mL) and heated at 160 °C for 3 h. The solvent was removed in vacuo and the resulting material was subjected to further derivatisation.

A portion of the hydrolysate mixture (300 μg) was dissolved in a solution of TEA/MeCN (2:3, 80 μL) and this solution was then treated with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) in 1:2 MeCN/$Me_2CO$ (1% solution, 75 μL). The vials were heated at 70 °C for 1 h, and the contents were neutralised with 0.2 N aq HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN/5% HCOOH

in $H_2O$ (1:1) and separated on a Jupiter C18 (15×4.60) by means of a linear gradient from 10% to 50% aq MeCN containing 5% formic acid and 0.05% trifluoroacetic acid, over 45 min at 0.15 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m*/*z* interval of 320–900) and the data were analyzed using the suite of programs Xcalibur; all masses were reported as average values. The capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

To determine the absolute configuration of Pro residue in perthamide G (**2**), authentic samples of Pro were used as standard. Retention times of L-FDAA-derivatized prolines: L-Pro (17.14 min) and D-Pro (18.76 min). Perthamide G (**2**) contains L-Pro (17.16 min).

All FDAA derivatives of amino acid residues in perthamides H (**3**) and I (**4**) were co-eluted with the corresponding ones in perthamides C and E.[13]

To determine the absolute configuration of 2-amino-2-(2,4-dioxooxazolidin-5-yl)acetic acid residue (ADAA) in perthamides J (**5**) and K (**6**), an authentic sample of perthamide C was used as a standard.

Perthamide C (**1**) contained (2*R*,3*S*)-β-OHAsp (10.20 min). Perthamides J and K contained (2*R*,3*S*)-β-OHAsp (10.02 min).

### 3.5. LC–MS on the crude *n*-BuOH extract

An aliquot of the crude *n*-BuOH extract was dried under vacuum, diluted with MeCN and separated on a Jupiter C18 column (25×1.8 mm i.d.) by means of a linear gradient from 10% to 95% aq MeCN containing 10 mM of ammonium acetate, over 45 min at 0.15 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m*/*z* interval of 950–1200) and the data were analyzed using the suite of programs Xcalibur; all masses were reported as average values. The capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

### 3.6. Carrageenan-induced mouse paw oedema

Male Swiss mice (CD-1; Harlan, Italy) weighing 28–30 g were separated in groups ($n$=6) and lightly anaesthetized with isoflurane. Each group of animals received subplantar injection of 50 μL of carrageenan 1% (w/v) or saline into the left footpad.[19] Paw volume was measured by using an hydropletismometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection and 2, 4, 6, 24, 48, 72 and 96 h thereafter. The same operator always performed the double-blind assessment of paw volume. The increase in paw volume was evaluated as the difference between the paw volume at each time point and the basal paw volume. Each group of animals received intraperitoneal administration of compounds **1**, **2**, **3**, **4**, **5**, **6** (0.3 mg/kg, 100 μL) or vehicle (PEG). All compounds were administrated immediately before carrageenan injection and 24 h thereafter.

### 3.7. Statistical analysis

Results were expressed as mean±SEM. Statistical analysis was determined by one way ANOVA followed by Dunnett's test for multiple comparisons, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when $p$ was less than 0.05.

## Acknowledgements

This work was supported by grants from MIUR (PRIN 2009) 'Sostanze ad attività antitumorale: isolamento da fonti marine, sintesi di analoghi e ulteriore sviluppo della chemoteca LIBIOMOL' Rome, Italy. NMR spectra were provided by the CSIAS, Centro Interdipartimentale di Analisi Strumentale, Faculty of Pharmacy, University of Naples. We thank the Solomon Islands government for collection permits, the Fisheries Department, R. Sulu (University of the South Pacific in Honiara) for their help and assistance, and Dr. John Hooper for the identification of the sponge.

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